Local Activation of the Innate Immune System in Buruli Ulcer Lesions

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Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is a chronic necrotizing disease of the skin and the underlying soft tissue. Fat tissue necrosis accompanied by minimal inflammation is considered the most reliable histopathologic feature of BU. There may be a constant influx of inflammatory cells to the sites of active infection but these are thought to be killed by mycolactone, a polyketide toxin produced by *M. ulcerans*, through apoptosis and necrosis. Here we describe the spatial correlations between mycobacterial load and the expression of dendritic cell (DC) surface markers (cluster of differentiation (CD)83, CD11c, and CD123), the Toll-like receptor (TLR) 9 and pro- and anti-inflammatory cytokines (IL-8, IL-6, tumor necrosis factor-alpha (TNF-α), IFN-α, IL-12p40, IL-10, and IFN-γ) within BU lesions. Although IL-8, IL-6, and TNF-α messenger RNA (mRNA) was detectable by real-time PCR in all lesions, the expression of the other cytokines was only found as small foci in some lesions. Correlations of the distribution of mRNA encoding the activation marker CD83 and the DC subset markers CD123 and CD11c indicate that both activated plasmacytoid and myeloid dendritic cells were present in the lesions. Results suggest that *M. ulcerans* specific immune responses may develop once therapeutic interventions have limited the production of mycolactone.

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INTRODUCTION

Buruli ulcer (BU) caused by *Mycobacterium ulcerans* is a chronic necrotizing disease of skin and soft tissue. Generally it manifests initially as firm, non-tender, subcutaneous nodules, probably at the sites of penetrating skin trauma (pre-ulcerative stage). Subsequently, these areas become fluctuant, followed by the formation of an ulceration with undermined edges (ulcerative stage). Ulcers can be extensive, involving more than 10% of the patient's skin surface (Johnson *et al.*, 2005). Subcutaneous fat is particularly affected, but underlying bone may also become involved in advanced cases. In BU lesions clumps of extracellular acid-fast bacilli surrounded by areas of necrosis are found. Fat tissue necrosis accompanied by minimal inflammation is considered the most reliable histopathologic feature of BU (Hayman and McQueen, 1985; Hayman, 1993; Guarner *et al.*, 2003). In late stages of the disease, intralesional influx of leukocytes and granulomatous responses in the dermis and panniculus has been described. If left untreated, spontaneous healing of BU lesions can occur after extended periods of progressive ulceration (Asiedu *et al.*, 2000). Traditionally, BU is treated by wide surgical excision, drug therapy has been considered ineffective, but recent data suggest that combinations of anti-mycobacterial antibiotics can support or replace surgical treatment (Etuaful *et al.*, 2005). Provisional World Health Organization (WHO) guidelines now recommend the use of rifampicin and streptomycin for the treatment of BU (http://www.who.int/buruli/information/antibiotics/en/index1.html).

M. ulcerans is unique among mycobacterial pathogens in that it is mainly extracellular and produces a plasmid-encoded toxin with a polyketide-derived macrolide structure, named mycolactone (Stinear *et al.*, 2004). Mycolactone is believed to play a central role in determining the extracellular localization of the bacteria and modulation of immunological responses to *M. ulcerans* (Adusumilli *et al.*, 2005). Observations in rodents experimentally infected with mycolactone producing and mycolactone-negative *M. ulcerans* strains suggested that inflammatory cells are rapidly killed by necrosis when encountering high toxin concentrations. Inflammatory cells more distant from the necrotic center are thought to be killed via apoptosis resulting in extracellular bacteria surrounded by an area of coagulation necrosis. In contrast, granulomatous lesions with strong self-healing tendencies were observed with mycolactone-negative mutants (Oliveira *et al.*, 2005).

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Abbreviations: BU, Buruli ulcer; CD, cluster of differentiation; DC, dendritic cell; mRNA, messenger RNA; P-DC, plasmacytoid dendritic cell; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-alpha

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Intrigued by the described lack of inflammatory responses in BU lesions, we have analyzed the impact of *M. ulcerans* infection on the activation of the skin innate immune system, including dendritic cells (DC). Here we describe the spatial correlations between bacterial load and the expression of DC-surface markers (cluster of differentiation (CD)83, CD11c, and CD123), the intracellular receptor Toll-like receptor (TLR)9 and pro- and anti-inflammatory cytokines (IL-8, IL-6, tumor necrosis factor-alpha (TNF-α), IFN-α, IL-12p40, IL-10, and IFN-γ) within BU lesions.

RESULTS

Quantitative real-time PCR was used to determine the spatial distribution of messenger RNA (mRNA) encoding cytokines and cell-surface markers of the innate immune system within surgically excised early ulcerative BU lesions of three selected patients. Histopathological changes and *M. ulcerans* DNA levels in the same tissue samples have been described previously (Rondini *et al.*, 2006). A summary of these data is provided in Figures 1 and 2 for direct comparison with the distribution of cytokine and DC marker mRNA.

Distribution of DC marker mRNA

Figure 1 shows the spatial pattern of mRNA encoding the cell-surface marker CD83, CD11c, CD123, and the intracellular receptor TLR9. Percent values normalized to β-actin mRNA are provided. In all three patients CD83 (Figure 1d–f) and CD123 mRNA (Figure 1j–l) was detectable along the entire lesions. Relative levels ranged from 0 to 4.2% (as compared to 0.2±0.1% in normal skin) and from 0 to 7.4% (0.2±0.2% in normal skin), respectively. CD11c (Figure 1g–i) and TLR9 mRNA (Figure 1m–o) showed a more focal distribution with relative levels ranging from 0 to 92% (0.4±0.2% in normal skin) and 0–1.2% (<0.01% in normal skin), respectively. For all four markers peak values were thus much higher than in normal skin. In many cases peaks were located close to foci of *M. ulcerans* DNA (sample G in patient A, samples D and F in patient B, and samples D and E in patient C).

Distribution of cytokine mRNA

Expression of cytokines with pro-inflammatory or anti-inflammatory activity was analyzed (Figure 2). IL-8, IL-6, and TNF-α mRNA was detectable in all three BU lesions, albeit in different amounts and in markedly different spatial patterns. Peaks of the relative levels of IL-8 mRNA were associated with the ulcerations and the histological detection of neutrophils (Figure 2a–c). In contrast, location of the relative peaks of IL-6 mRNA with respect to the location of ulcerations and peaks of *M. ulcerans* DNA varied markedly between lesions. In patient A, the relative IL-6 mRNA levels were highest at the less affected borders of the excised tissue, in patient B it was peaking at the nodular pre-ulcerative lesion and in patient C at and around the ulceration (Figure 2d–f). TNF-α mRNA was broadly distributed over the lesion. Relative peak levels of IL-8, IL-6, and TNF-α mRNA (130.395, 10, and 2.3%, respectively) were dramatically higher than the levels found in normal skin (4.7±5.1, 0.1±0.1, and 0.1±0.1%, respectively).

Like in normal skin, IL-12p40 mRNA levels were below the detection limit in all three BU lesions analzsed (<0.03%) (data not shown). In contrast, IFN-α, IFN-γ, and IL-10 mRNA, also undetectable in normal skin, was found at least in one of the three analyzed lesions in spatially highly restricted foci. IL-10 and IFN-γ mRNA was detected only in patient B (Figure 2n). Although a peak of IL-10 mRNA was associated with the secondary non-ulcerated nodule (peak value 1.2%), IFN-γ mRNA was found in one sample close to the small ulceration and the associated granulomas (peak value 0.2%) (Figure 2n). Significant levels of IFN-α mRNA were primarily found in patient C (Figure 2j–l), peaking towards the right margin of the excised tissue (Figure 2l, peak value in sample I, 246%).

Correlations of the spatial distribution of mRNA species

The spatial mRNA distributions of the two DC subset markers CD11c and CD123 were positively correlated with that of the cellular maturation marker CD83 (CD83 *vs* CD123: $r=0.63$, $P<0.0001$; CD83 *vs* CD11c: $r=0.54$, $P=0.001$; Figure 3a and b). Also strong positive correlations of the distribution of CD83 with IL-6 and TNF-α were observed ($r=0.67$, $P<0.0001$, and $r=0.80$, $P<0.0001$, respectively; Figure 3c and d). Correlation of IL-6 expression with CD123 was tighter ($r=0.66$, $P<0.001$; Figure 3e) compared with CD11c ($r=0.41$, $P=0.02$; Figure 3f). The correlation of both DC subset markers with TNF-α expression was moderate (CD11c *vs* TNF-α: $r=0.50$, $P=0.002$; CD123 *vs* TNF-α: $r=0.48$, $P=0.004$; data not shown). There was no indication of a correlation between IFN-α and CD123, CD83, or CD11c (data not shown).

Immunohistochemical detection of CD123 and TLR9-positive cells

Results of quantitative real-time PCR and immunohistochemistry were highly associated, that is the relative numbers of CD123 and TLR9-positive cells were consistent with the mRNA levels detected by real-time PCR (Figure 4). In the thin sections of lesions positive for CD123 and TLR9 mRNA, CD123, and TLR9 antibodies stained cells with plasmacytoid features (inset Figure 4b and d).

DISCUSSION

Under homeostatic conditions, cutaneous DCs include epidermal Langerhans cells and interstitial/dermal DCs that are of myeloid origin (Kupper and Fuhlbrigge, 2004). Our real-time PCR and immunostaining data indicate that in addition to the CD11c-positive CD123-negative myeloid DCs (Colonna *et al.*, 2004), CD123-positive plasmacytoid DCs (P-DC), are present in early ulcerative lesions. P-DC are of lymphoid origin (Colonna *et al.*, 2004), CD11c-negative and known to be recruited to diseased skin in conditions such as systemic lupus erythematosus, atopic dermatitis, psoriasis vulgaris, and contact dermatitis (Wollenberg *et al.*, 2002; Bangert *et al.*, 2003; Nestle *et al.*, 2005).

One of the surface molecules upregulated upon DC activation and maturation is CD83 (Lechmann *et al.*, 2002).

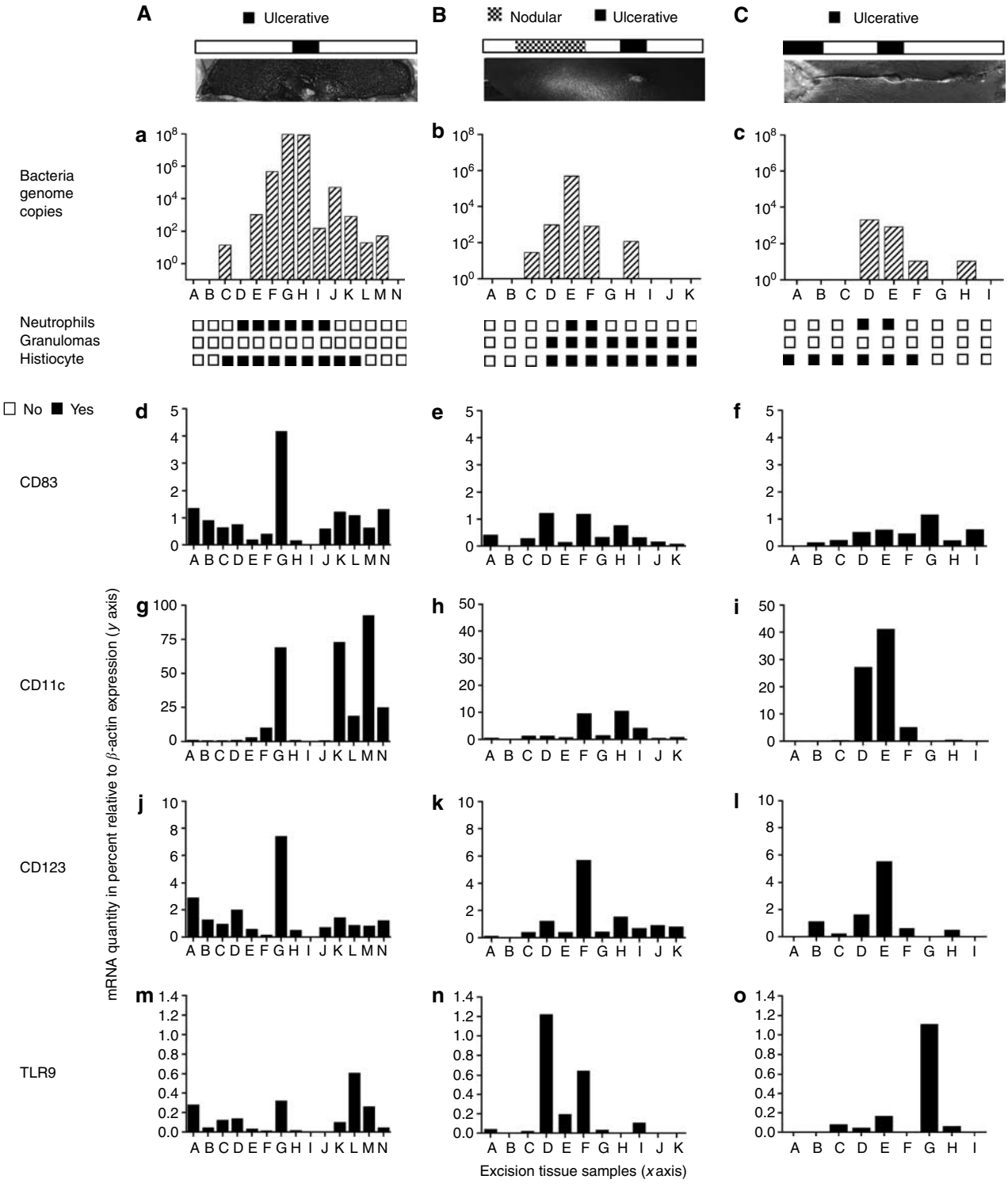

Figure 1. Spatial localization of bacterial load and DC markers in BU lesions. Patients A, B, and C excisions with tissue samples: A–N, A–K, A–I; respectively. Relative quantity of mRNA for the surface markers (**d–f**) CD83, (**g–i**) CD11c, (**j–l**) CD123, and (**m–o**) intracellular receptor TLR9 expressed in percent relative to β-actin gene expression. (**a–c**) *M. ulcerans* DNA load and histopathological changes of the excisions (Rondini *et al.*, 2006).

Although CD83 is also expressed on activated human B and T cells and a subpopulation of activated monocytes (Lechmann *et al.*, 2002), the observed correlations of mRNA expression between CD83 and CD123, or CD11c indicated, that both P-DC and myeloid DC were activated in the BU lesions. The distribution of mRNA encoding the highly expressed pro-inflammatory cytokines IL-6 and TNF-α was also strongly correlated with the activation marker CD83. Expression of

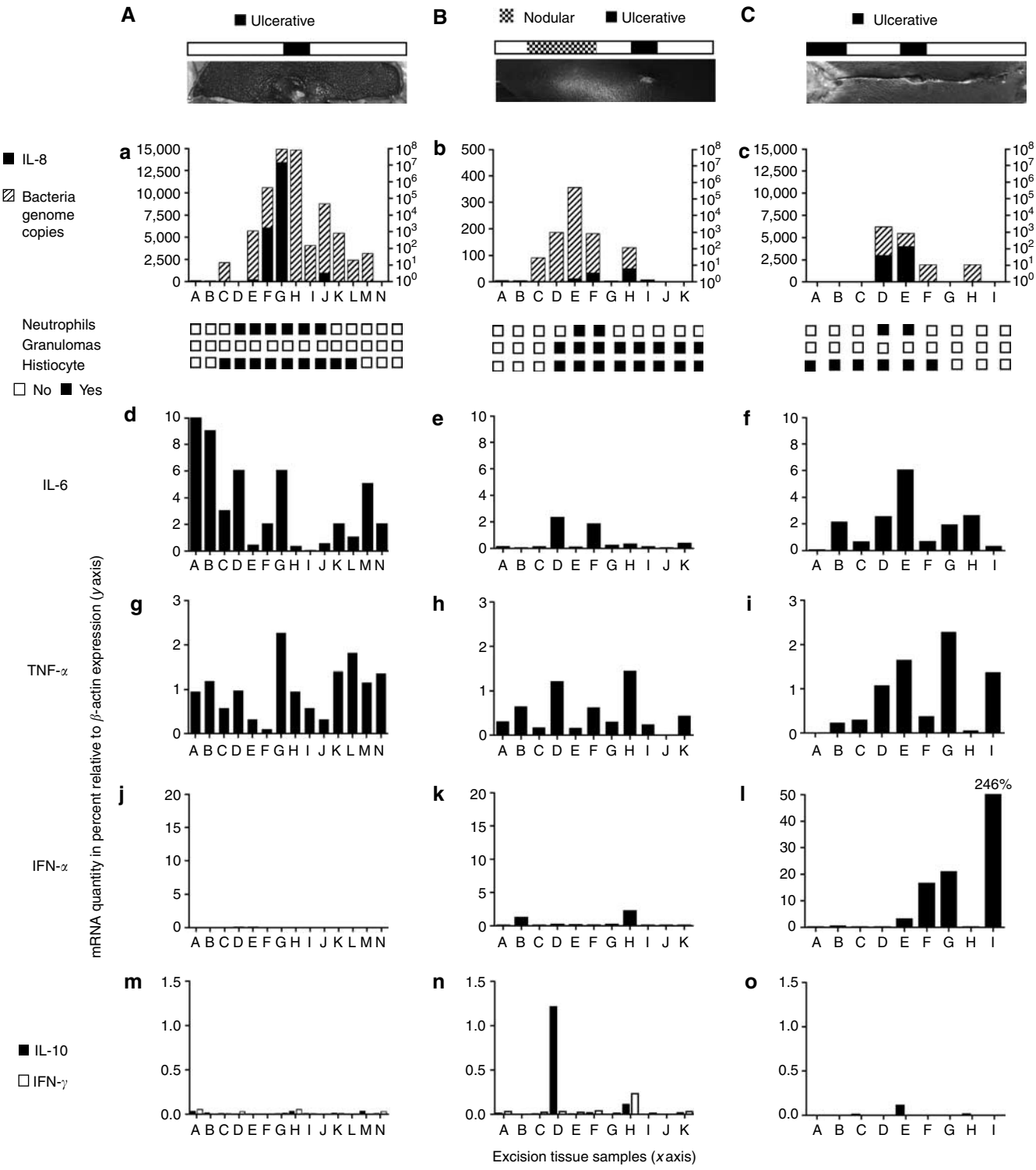

Figure 2. Distribution of bacterial load and cytokine mRNA in BU excisions. Patient A, B, and C excisions with tissue samples: A–N, A–K, A–I; respectively. Relative quantity of mRNA for the cytokines (**a–c**) IL-8, (**d–f**) IL-6, (**g–i**) TNF-α, (**j–l**) IFN-α, (**m–o**) IL-10 and IFN-γ expressed in percent relative to β-actin gene expression. (**a–c**) *M. ulcerans* DNA load and histopathological changes of the excisions (Rondini *et al.*, 2006).

IL-6 was additionally strongly correlated with that of CD123, indicating that activated P-DC may represent the major source of IL-6 expression in the BU lesions. In contrast to myeloid DC, P-DC express TLR7 and TLR9 but lack TLR2, TLR3, TLR4, and TLR5. In the majority of patient samples analyzed, expression of TLR9 and CD123 mRNA was consistent, supporting the presence of P-DC in BU lesions (Figure 1). Signalling through TLR7 and TLR9 results in P-DC

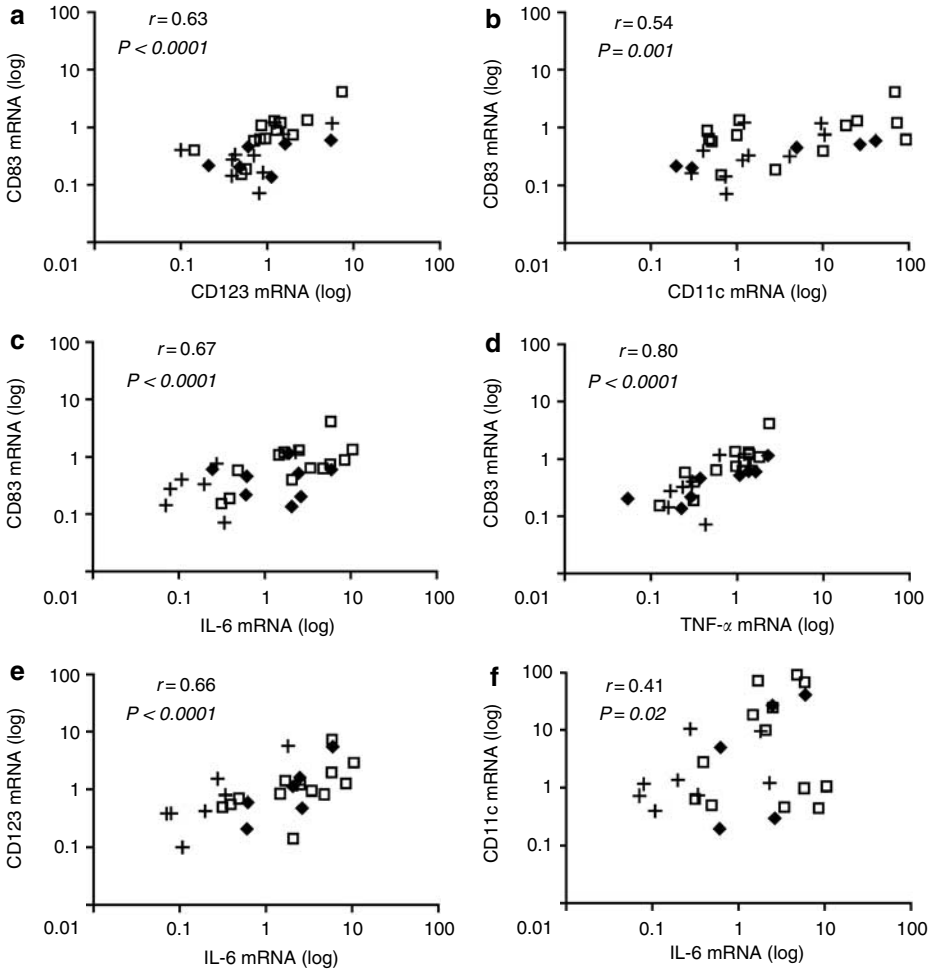

Figure 3. Correlations of DC markers and cytokine expression. Patient A (●), B (♦), and C (+). Spearman's correlations of the mRNA spatial distribution in the excisions are given. (**a**) CD123 versus CD83; (**b**) CD11c versus CD83; (**c**) IL-6 versus CD83; (**d**) TNF-α versus CD83; (**e**) IL-6 versus CD123; (**f**) IL-6 versus CD11c. Spearman's correlations with an r ranging from 0.4 to 0.6 and P-value of <0.05 are moderate positive; with $r>0.6$ and $P<0.05$ are positive to strong positive. Each point represents the values of one tissue sample.

activation to secrete large amounts of type I IFN and moderate amounts of TNF-α and IL-6 (Colonna *et al.*, 2004). In contrast to IL-6, no correlation between CD123 and IFN-α mRNA was observed. IFN-α expression by P-DC seems to be variable; whereas P-DC activation by TLR9 in response to viruses results in secretion of large amounts of IFN-α (Colonna *et al.*, 2004), during the development of psoriatic phenotype IFN-α expression by P-DC seems to be only an early and transient event (Nestle *et al.*, 2005). Consistent with published data (Prevot *et al.*, 2004; Kiszewski *et al.*, 2006; Phillips *et al.*, 2006), TNF-α and IL-8 mRNA levels were, like those of IL-6 and IFN-α mRNA, much higher in the BU lesions than in normal skin. As moderate correlation between DC markers and TNF-α mRNA was observed, TNF-α mRNA expression may be in part associated with other cell types, like monocytes, activated T cells, or natural killer cells.

The mechanism of immune protection in *M. ulcerans* remains unclear. Evidence from genetic defects in the IFN-γ signalling pathway supports the role of IFN-γ in protection against a range of non-tuberculous mycobacterial disease, including *M. ulcerans* (Ottenhoff *et al.*, 2005). Peripheral blood mononuclear cells from BU patients with active disease showed significantly reduced lympho-proliferation and IFN-γ production in response to stimulation with live or dead *M. bovis Bacillus* Calmette-Guérin, *M. ulcerans*, purified protein derivative of *M. tuberculosis*, isopentenyl pyrophosphate, and non-mycobacterial antigens like reconstituted influenza virosomes (Gooding *et al.*, 2001, 2002, 2003; Prevot *et al.*, 2004; Yeboah-Manu *et al.*, 2006). Prevot *et al.* (2004) showed with semiquantitative PCR analyses that the systemic Th1 downmodulation was mirrored by local, intralesional cytokine profiles. High IFN-γ with low IL-10 mRNA levels were present in early, nodular lesions, and low IFN-γ mRNA levels were detected in late ulcerative lesions (Prevot *et al.*, 2004). Hence, in active *M. ulcerans* disease, the Th1 response seemed to be downregulated both locally and systemically. The presence of IL-6 during T-cell priming may promote Th2 differentiation and simultaneously inhibit Th1 polarization (Diehl and Rincon, 2002). Therefore the close association of CD123 (P-DC) with IL-6 in conjunction with the lack of IFN-α production may favor Th2 development and

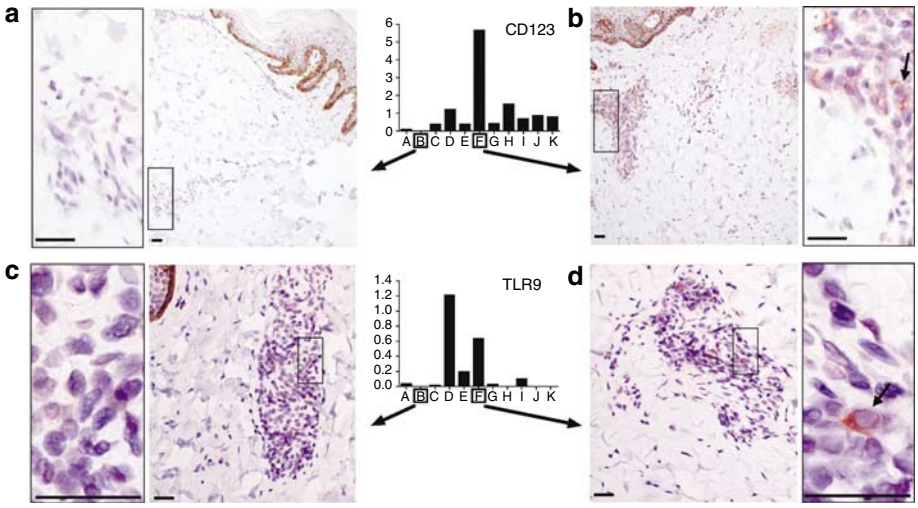

Figure 4. Immunohistochemical analysis of P-DC recruitment to BU excisions. Immunohistochemical stainings in thin sections of tissues samples B and F of patient B. (**a**, **b**) CD123 staining (original magnification ×100, inset original magnification ×400) and (**c**, **d**) TLR9 staining (original magnification ×200, inset original magnification ×1,000), bars = 40 μm.

result in the observed Th1 downmodulation in BU. IL-6 is a pleiotropic cytokine involved in the growth and differentiation of numerous cell types, including those of dermal and epidermal origin (Paquet and Pierard, 1996). In the skin, it is induced in a broad range of dermatotoxic reactions and may be involved in wound healing (Hernandez-Quintero *et al.*, 2006). The presence of high numbers of P-DC in the lesions in the absence of IFN-α gene expression raises also the issue of a tolerogenic role of these cells, as suggested in primary cutaneous melanomas (Vermi *et al.*, 2003).

IL-10 and IFN-γ mRNA was detected in one of the three analyzed BU lesions, where it was present only in highly focal areas. Phillips *et al.* (2006) showed wide variations in IL-10 and IFN-γ mRNA expression among individual skin punch biopsies. Generally, our results demonstrate that expression of cytokines and cell-surface markers can vary considerably within a BU lesion. Therefore, results obtained with biopsies of BU lesions do not necessarily reflect the overall profile of a lesion. Our comparison of the spatial relationship between bacterial load, DC marker, and pro-inflammatory cytokine mRNA suggests that the presence of clusters of *M. ulcerans* does not exclude innate immune system recruitment to the site of infection. This conclusion is consistent with the hypothesis of Oliveira *et al.* (2005) suggesting a constant influx of neutrophils, monocytes/macrophages, and lymphocytes to active *M. ulcerans* lesions. Potentially, *M. ulcerans* specific immune responses may therefore develop, once a therapeutic intervention, such as a successful antibiotic treatment, is limiting the production of mycolactone.

MATERIALS AND METHODS

Clinical specimens

Three BU patients with ulcerative lesions, who received standard treatment at the Amasaman Health Centre in the Ga district in Ghana, were enrolled in this study. The standard treatment comprised wide surgical excision including margins of macroscopically healthy tissue followed by skin grafting. BU clinical diagnosis was reconfirmed by IS2404 PCR, microscopic detection of acid-fast bacilli, and observation of characteristic histopathological changes. The distribution of *M. ulcerans* DNA and histopathological examination within the excised tissue samples analyzed here, have been described elsewhere (Rondini *et al.*, 2006). Patient A presented with an ulcerated plaque (ulcer size 4 × 5 cm) on the dorsal aspect of the left upper arm. The central necrotic slough was associated with typical inflammatory cells whereas no granulomas were seen in any zone of the excision. The highest mycobacterial DNA burden was present at the base of the ulcer and decreased towards the margins of the excision (Figure 1a). Patient B presented with a small-ulcerated lesion (∅ 1 cm) and a larger non-ulcerated nodule located about 3 cm apart on the dorsal aspect of the right elbow. The *M. ulcerans* DNA was present with high load within the non-ulcerated nodule and, with lower load, in the ulcerated region. Between these two lesions no bacterial DNA was detected. Granulomas were present across the tissue from nodule to ulcer till the right margin of the excision (Figure 1b). Patient C presented with a small ulcer 5 cm away from a larger ulcer, which was surrounded by scar tissue and showed evidence of previous treatment. *M. ulcerans* DNA was only present at the base of the small ulcer and no granulomas were detected in the whole excision (Figure 1c). Ethical approval for analyzing patient specimens was obtained from the local ethical review board of the Noguchi Memorial Institute for Medical Research and participants gave their written informed consent. The study was conducted according to the Declaration of Helsinki Principles.

RNA extraction, removal of genomic DNA, and reverse transcription

RNA was extracted from several samples of equal size, each comprising skin and fat tissue, which were obtained from BU patients' excised lesions: patient A, 14 samples (A–N); patient B, 11 samples (A–K); patient C, nine samples (A–I). Samples were disrupted

by sonication for 2 minutes (Sonifer® Branson 250, Branson Ultrasonics Corporation, Danbury, CT) and centrifuged for 3 minutes at 10,000 × *g*. RNA was extracted from the tissue lysate using the RNeasy Mini Kit (Qiagen AG, Basel, CH) and treated with DNase I (Invitrogen, Paisley, UK) to remove genomic DNA.

To synthesize complementary DNA, total RNA was incubated with oligo d(T) for 10 minutes at 65°C, and put on ice. A reaction mixture containing dNTP mix (125 nM), dithiothreitol (10 mM) and reverse transcriptase Moloney murine leukemia virus (200 U) with corresponding first strand buffer was added (Invitrogen, Paisley, UK). The reaction mix was incubated for 60 minutes at 37°C before enzyme inactivation for 5 minutes at 94°C.

Quantitative real-time polymerase chain reaction

Gene transcription was evaluated using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers and probes for six human cytokines (IL-6, IL-8, IL-10, IL-12p40, TNF-α, IFN-α, and IFN-γ), cell-surface proteins (CD11c, CD83, CD123, and TLR9) were used to amplify specific complementary DNA in duplicate, according to the manufacturers instructions (Applied Biosystems, Foster City, CA). The β-actin gene was used as an internal house keeping gene reference. Primers and probes for β-actin, IL-8, IL-12p40, IFN-α, CD11c, CD83, CD123, and TLR9 were obtained from Applied Biosystems. Primer and probes for IL-6 (Hartwig *et al.*, 2002), IL-10 (Giulietti *et al.*, 2001), TNF-α (Razeghi *et al.*, 2001), and IFN-γ (Kammula *et al.*, 1999) were synthesized by Mycrosynth (Balgach, CH). Having verified that the amplification dynamic remains proportional at all tested dilutions, RNA expression of each surface marker and cytokine was presented as percentage relative to β-actin gene expression. The assays were run in duplicates and the results with a standard deviation >2% were excluded. Correlation analyses were performed in Prism using the Spearman rank correlation coefficient. Mean values of surface markers and regulatory cytokines in six samples of healthy skin tissue are as follow: CD11c (0.4±0.2%), CD123 (0.2±0.2%), CD83 (0.2±0.1%), TLR9 (<0.01%), TNF-α (0.1±0.1%), IL-6 (0.1±0.1%), and IL-8 (4.7±5.1%). IFN-α, IFN-γ, IL-10, and IL-12p40 mRNA were undetectable.

Immunohistochemistry

Tissue samples were fixed overnight in neutral buffered 4% paraformaldehyde, embedded in paraffin according to standard protocol and cut into 5 μm sections using a microtome. After deparaffinization, sections were re-hydrated through graded alcohols and washed in distilled water. Antigen retrieval was performed by microwave unmasking technique in 10 mM EDTA pH 8.0. Subsequently, endogenous peroxidase was blocked with 0.3% H_2O_2 for 30 minutes and unspecific binding prevented by incubating with blocking serum for 20 minutes at room temperature. CD123 (clone 6H6) and TLR9 (clone eB72-1665) antibodies (eBiosciences, San Diego, CA) were diluted 1:100 and 1:1,000, respectively, in phosphate-buffered saline plus 0.1% Tween-20 and slides incubated in a humid chamber for 1 hour at room temperature. Sections were incubated for 30 minutes at room temperature with the secondary antibody biotin labelled (Vector Laboratories; 1:200 in phosphate-buffered saline). Slides were then labelled with streptavidin horseradish peroxidase conjugate (Vector Laboratories, Vectastain Elite ABC kit) for 30 minutes at room temperature and staining was performed by using Vector NovaRed and hematoxylin (counter stain).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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